Research Article

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Phenols in spikelets and leaves of field-grown oats (*Avena sativa*) with different inherent resistance to crown rust (*Puccinia coronata* f. sp. *avenae*)

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Abstract

BACKGROUND: Avenanthramides, health-beneficial phenols in oats, are produced in response to incompatible races of the crown rust fungus, *Puccinia coronata*, in seedlings of greenhouse-grown oats. This study aimed to elucidate whether avenanthramides and/or other phenolic compounds, together with the activities of phenylalanine ammonia lyase (PAL), phenoloxidase (PO) and the avenanthramide biosynthetic enzyme hydroxycinnamoyl-CoA : hydroxyanthranilate-*N*-hydroxycinnamoyl transferase (HHT), are associated with crown rust infection in mature field-grown oats. Nine oat (*Avena sativa* L.) genotypes with wide variation in crown rust resistance were exposed to naturally occurring fungal spores during the growth period.

RESULTS: In the spikelets avenanthramides as well as HHT activities were more abundant in the crown rust resistant genotypes, whereas *p*-coumaric and caffeic acids were more abundant in the susceptible ones. In the leaves avenanthramides were not associated with resistance. Instead two unknown compounds correlated negatively with the rust score. Phenols released by alkaline hydrolysis and PAL and PO activities were not related to rust infection, either in spikelets or in the leaves.

CONCLUSION: Because grains of crown rust-resistant oat genotypes seemed to have higher endogenous levels of health-promoting avenanthramides, use of resistant oats may contribute to a food raw material with health-beneficial effects.

Keywords: avenanthramides; crown rust; oats; phenols; spikelets; rust resistance

INTRODUCTION

Epidemiological studies indicate that a high intake of whole-grain foods with intact bran and germ may reduce the risk of contracting various chronic diseases such as diabetes[1] and cardiovascular disease.[2] Little is known of the mechanisms behind these beneficial effects, but as certain diseases are associated with free radical production and inflammatory processes,[3] low-molecular-weight phenolic compounds with anti-oxidative and/or anti-inflammatory activities may contribute to the health-promoting effects.

Oats (*Avena sativa* L.) contain a wide range of phenolic compounds that exist either in free or bound forms, mainly in the bran fraction.[4] Generally, the free phenolic compounds are cinnamic acid and its derivatives, whereas the bound phenolic compounds mainly are oxidatively coupled dimers of *p*-coumaric and ferulic acids, which are ester-linked to cell wall polymers. Avenanthramides, which are amides of anthranilic acid and cinnamic or avenalumic (5-phenyl-penta-2,4-dienoic) acid derivatives, are exclusively reported from oats, and they have been isolated from both grains[5–8] and leaves.[9,10] More than 15 different free avenanthramides are known to exist in the grains,[5] of which 2p, 2c and 2f (Fig. 1) constitute the three main ones.[11] In the leaves 2p and 2f are the major ones.[12] Recently also dimeric structures of avenanthramides have been identified in leaf tissues.[13,14] Avenanthramides are related to fresh taste of oats,[15] and some of them are shown to function as antioxidants *in vitro*[7,11,16] and *in vivo*.[17–19] Some are also believed to possess anti-inflammatory properties.[20–23] Lignans belong to the group of phytoesterogens that are believed to exhibit several health beneficial properties.[24] Recently, an oat-specific lignan, 4,4′-dihydroxy-3,3′-dimethoxy β-truxinic acid, esterified to sucrose, named tase, was identified in oat groats.[25] Biological effects of this lignan are not yet elucidated, but sucrose esters of similar truxinic acid derivatives are suggested to be anti-inflammatory compounds.[26] Free cinnamic acid derivatives and their dimers also exert several activities related to oxidative and inflammatory processes.[27–29]

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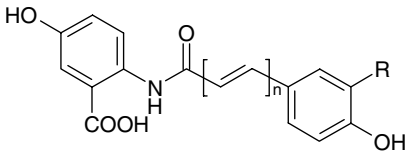

Avenanthramide	R	n
2p	H	1
2c	OH	1
2f	OCH_3	1
$2p_d$	H	2
$2f_d$	OCH_3	2

Figure 1. Structures of selected avenanthramides.

It is well known that the amounts of various phenols and activities of the enzymes phenylalanine ammonia lyase (PAL) and phenoloxidase (PO) increase in plants as a response to wounding, infection or mechanical damage.[30–32] PAL is a key enzyme in the biosynthesis of phenolic compounds, and PO mediates oxidative reactions of phenols, which are assumed to be involved in defence responses against fungal infections. Crown rust (*Puccinia coronata* f. sp. *avenae*) is the most common pathogen infecting oats.[33] In leaves of oat seedlings grown in a growth chamber certain phenolic compounds, including avenanthramides, increase as a response to incompatible races of crown rust fungi or the presence of other elicitors.[9,34] Also, activities of peroxidase, PAL and the avenanthramide biosynthetic enzyme, hydroxycinnamoyl-CoA : hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT), increase concurrently with the increase in avenanthramides. It has been demonstrated that labelled anthranilate, phenylalanine and *p*-coumaric acid are incorporated into avenanthramides after elicitation.[10,12,35,36] Studies have also shown that the content of the avenanthramides, in some cases along with the activity of HHT, increases in grains during germination.[37–39] However, when oat grain flour is steeped in water, the content of the avenanthramides decreases,[40] probably a result of oxidation by peroxidase[41] or PO (unpublished data). Oxidation of avenanthramides has been detected in flours from different genotypes of oats.[40]

In a recent study[42] it was shown that the content of avenanthramides increased gradually in spikelets and leaves during the development of nine field-grown oat genotypes and that avenanthramides were evident already at 3–5 days after heading in both tissues. It was also shown that HHT activity increased in the spikelets along with the development. In that study it was not evaluated whether the content of avenanthramides and activity of HHT were related to crown rust infection.

The aim of the present study was to explore whether the content of avenanthramides as well as other phenolic compounds and the activities of PAL, PO and HHT in spikelets and leaves of the nine field-grown oat genotypes were related to acquired crown rust infection at maturity. The genotypes had different inherent levels of resistance to the crown rust fungi, ranging from fully resistant to completely susceptible.

MATERIAL AND METHODS

Oat materials

Nine oat genotypes (*Avena sativa* L.) that were adapted to the midwestern USA and had a wide variation in their resistance to the crown rust fungus *Puccinia coronata* (Table 1) were grown in 2001 on the West Madison Farm, Madison, Wisconsin, USA.[42] They were planted in a randomized complete block design with three replications that consisted of blocks with four 3 m rows, 0.3 m apart. The plants were exposed to naturally occurring spores of the crown rust fungus, and the rust score was recorded 86 days after planting. Scores from 0 to 100 indicate no rust to complete coverage, respectively. Crown rust infestation was moderately severe in 2001, and the genotypes were well differentiated in their resistance to the fungal attack. As expected, Bay was severely infested, and Ogle and Dane also took on significant rust. Jim, Gem, Vista and Moraine showed an intermediate level of resistance, whereas Belle and X7571-1 showed only a trace or no pustules (Table 1). Spikelets and leaves were harvested seven times at approximately 5-day intervals from heading (Table 1) to maturity, and immediately kept on ice until freezing (*ca* 1–2 h after harvest). The samples were freeze-dried, and then stored in paper bags at room temperature until milling and extraction. For this study, the samples from the last harvest of leaves (25–28 days after heading) and spikelets (31–34 days after heading) were analysed.

Table 1. Number of days elapsed from planting[a] to heading and rust scores 86 days after planting of nine different oat genotypes classified from inherent susceptibility (s) to resistance (r) towards crown rust infection

Genotypes	Grade of inherent resistance	Heading (days from planting)	Rust score[b] (%)
Bay	Susceptible (s)	72	75, 75, 85
Ogle	Susceptible (s)	68	45, 50, 45
Dane	Intermediate susceptible (is)	63	35, 40, 45
Jim	Intermediate susceptible (is)	65	25, 25, 35
Gem	Intermediate resistant (ir)	69	12, 18, 20
Vista	Intermediate resistant (ir)	72	15, 18, 18
Moraine	Intermediate resistant (ir)	65	12, 15, 15
Belle	Resistant (r)	72	2, 0, 1
X7571-1	Resistant (r)	72	0, 0, 0

[a] Planting day was 18 April.
[b] Replicates 1, 2 and 3, respectively.

Chemicals

p-Coumaric, ferulic, caffeic acids and DL-tryptophan were purchased from Sigma Chemical Co. (St Louis, MO, USA), cinnamic acid and 5-hydroxyanthranilic acid from Aldrich Chemie (Steinheim, Germany), and dimethylsulfoxide (DMSO), 1,4-dithiothreitol (DTT), L-phenylalanine and tris-(hydroxymethyl)-aminomethane were purchased from Merck (Darmstedt, Germany). *p*-Coumaroyl-CoA (*p*-CoA) was synthesized according to the method described by Peterson and Dimberg.[42] Synthetic avenanthramides *N*-(4′-hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2p), *N*-(4′-hydroxy-3′-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2f) and *N*-(3′,4′-dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2c) were provided by Dr K Sunnerheim, Department of Natural Sciences, Mid Sweden University, Sundsvall, Sweden. The lignan, 4,4′-dihydroxy-3,3′-dimethoxy β-truxinic acid, esterified to sucrose (tase), was previously purified from oat groats.[25] All other chemicals and solvents used in the study were provided from Merck, and they were of analytical grade and used without further purification.

Extraction of free and alkaline-released compounds from oat spikelets and leaves

Freeze-dried spikelets or leaves were ground in a Retsch ZM-1 mill (Haan, Germany) to pass a 0.5 mm sieve. Duplicate 0.5 g aliquots of each sample were extracted three times with 10 mL 80% aq. EtOH at 50 °C for 10 min in a shaking water bath. The samples were centrifuged at 2500 rpm (Multifuge 3S/3S-R, Heraeus, Kendro Laboratory Products Langenselbold, Germany) for 5 min and the supernatants combined and dried *in vacuo* at 40 °C. Residues of the spikelet samples were dissolved in 1 mL MeOH and filtered through a 0.45 μm GHP Acrodisc 13 filter (Pall Co, Ann Arbor, MI, USA) before analysis by high-performance liquid chromatography (HPLC) (see below). To eliminate the chlorophyll, the leaf residues were dissolved in 2 mL 40% aq. MeOH and filtered through a 3 mL C18 Bond Elute (Varian, Palo Alto, CA, USA) column rinsed with MeOH (three column volumes) and equilibrated with 40% MeOH. The samples were eluted with 10 mL of 40% MeOH, and the eluates were collected and vacuum dried at 40 °C. These residues were dissolved in 1 mL of 40% MeOH and analysed by HPLC (see below). The samples were also diluted 10-fold with MeOH and reanalysed by HPLC to obtain area values for the largest peaks.

The pellets left after the 80% aq. EtOH extraction were washed three times with 10 mL distilled H_2O at 50 °C for 10 min in a shaking water bath, followed by centrifugation at 2500 rpm for 5 min. Next, the pellets were washed three times with 10 mL acetone at room temperature for 10 min. After centrifugation at 2500 rpm for 5 min the supernatants were discarded.

To hydrolyse these pellets, 20 mL of 1 mol L^{-1} KOH in MeOH (for spikelets) or 3 mol L^{-1} aq. KOH (for leaves) were added. The samples were incubated on a roller mixer (Swelab mixer 820, Stockholm, Sweden) at room temperature (20 °C) for 16 h. The samples were acidified with 4 mL of 6 mol L^{-1} HCl and then centrifuged at 2500 rpm for 5 min. The spikelet sample supernatants were collected and dried *in vacuo* at 40 °C and the residues dissolved in 2 mL MeOH for HPLC analyses. The leaf sample hydrolysates were shaken three times with 50 mL EtOAc. The EtOAc phases were removed and combined, and then evaporated *in vacuo* at 40 °C. The residues were dissolved in 1 mL MeOH and filtrated using 0.45 GHP Acrodisc 13 filters before HPLC analyses. The peak areas per gram freeze-dried oat sample were calculated and used in the statistical analyses for all peaks.

Isolation and identification of avenanthramides $2p_d$ and $2f_d$ and the flavone tricin

The compounds corresponding to S19, S20 and S21 in the HPLC chromatogram of the non-hydrolysed spikelet samples (Fig. 2(a)) were isolated and identified. The ethanol extracts (see above) were pooled and the compounds purified using solid-phase (Bond Elute C18 cartridge) extraction and preparative thin-layer (silica gel; 254 nm fluorescence indicator) chromatography methods. Every step in the purification process was monitored by HPLC analyses. The structures of the purified compounds were elucidated by ^{1}H-nuclear magnetic resonance (NMR) and 2D-NMR analyses on a Bruker DRX-400 (400 MHz) instrument (Rheinstetten, Germany) and by molecular weight determination using liquid chromatography–mass spectrometry (LC-MS) (Agilent 1100 LC/MSD system, Santa Clara, CA, USA)). S19 and S20 were found to be the avenanthramides *N*-(4′-hydroxy-(E)-avenalumoyl)-5-hydroxyanthranilic acid ($2p_d$) and *N*-(4′-hydroxy-3′-methoxy-(E)-avenalumoyl)-5-hydroxyanthranilic acid ($2f_d$), respectively (Fig. 1). S21 corresponded to the flavone tricin (tri). The identities of $2f_d$ and tricin were also confirmed by comparison with literature data.[5,43]

Enzyme assays

The method used for detection of PAL, PO and HHT activities were modified versions of methods described by Carver *et al*.[44] and Bryngelsson *et al*.[37,40] The enzymatic activities were measured with the oat matrix present during the assay instead of in a soluble extract. This was done to simplify the method but also to mimic the assay used for PO activity, which in oat kernels is only possible to detect if the oat matrix is present during the assay (unpublished data). To avoid activity of the PO enzymes in the PAL and HHT assays, the reducing agent DTT was added.[40] The amount of oat flour and buffer used and the incubation times chosen were calculated to obtain suitable enzyme activities.

Oat groats were obtained by manually dehulling the spikelets. The groats and the leaves were milled (Retsch ZM-1 mill) just prior to analysis of the different enzyme activities.

Phenylalanine ammonia-lyase (PAL) activity

Quaduplicate aliquots of the flours (30 mg) were incubated for 20 min at room temperature with 500 μL 50 mmol L^{-1} tris-bis buffer (pH 8.5, 10% DTT). As controls, 100 μL 6 mol L^{-1} HCl was added to two of the aliquots to inactivate the enzymes, and then 2.5 mL of 0.2% phenylalanine solution was added to all four aliquots. They were vortexed and incubated in a water bath for 3 h at 36 °C. To the two active aliquots 100 μL of 6 mol L^{-1} HCl was added. All four aliquots were then vortexed, and 2 mL was transferred to microfuge tubes and centrifuged at 13 100 rpm (Eppendorf centrifuge 5417C, Hamburg, Germany) for 10 min. The supernatants were transferred to vials and analysed by HPLC (see below). The increase in the concentration of cinnamic acid (nmol g^{-1} h^{-1}) was used as a measure of PAL activity.

Monophenoloxidase (PO) activity

Sodium phosphate buffer, 450 μL (0.1 mol L^{-1}, pH 7.0) was added to five 25 mg aliquots of the different flours. To three of the five aliquots, 50 μL of 10 mmol L^{-1} *p*-coumaric acid, dissolved in methanol, was added, and the samples were incubated at 30 °C in a water bath for 2 h. The reaction was stopped by adding 100 μL glacial acetic acid. As a blank aliquot, 100 μL acetic acid was added to the other two aliquots prior to the substrate addition to inactivate the enzyme. The volumes were adjusted to 2.5 mL with MeOH, and they were vortexed. Approximately 2 mL of the supernatant was removed to microfuge tubes and centrifuged at 13 000 rpm for 5 min and subjected to HPLC analyses. To confirm that no detectable endogenous *p*-coumaric acid was present in the oat flour, a second control was included where no exogenous *p*-coumaric acid was added to the assay tubes. The decreased amount of *p*-coumaric acid (nmol g^{-1} h^{-1}) compared to the blank sample was used as a measure of PO activity. Caffeic acid, the product formed in the oxidation reaction, was not used in the calculation of the PO activity, as this compound was further oxidized in the assay.

Hydroxycinnamoyl-CoA : hydroxyanthranilate N-hydroxycinnamoyl transferase (HHT) activity

Flours (25 mg) were weighed into microfuge tubes (triplicates) and 200 μL of 100 mmol L^{-1} tris- bis buffer (with 2 mmol L^{-1} DTT), pH 7.3, was added. Ten microlitres of 10 mmol L^{-1}

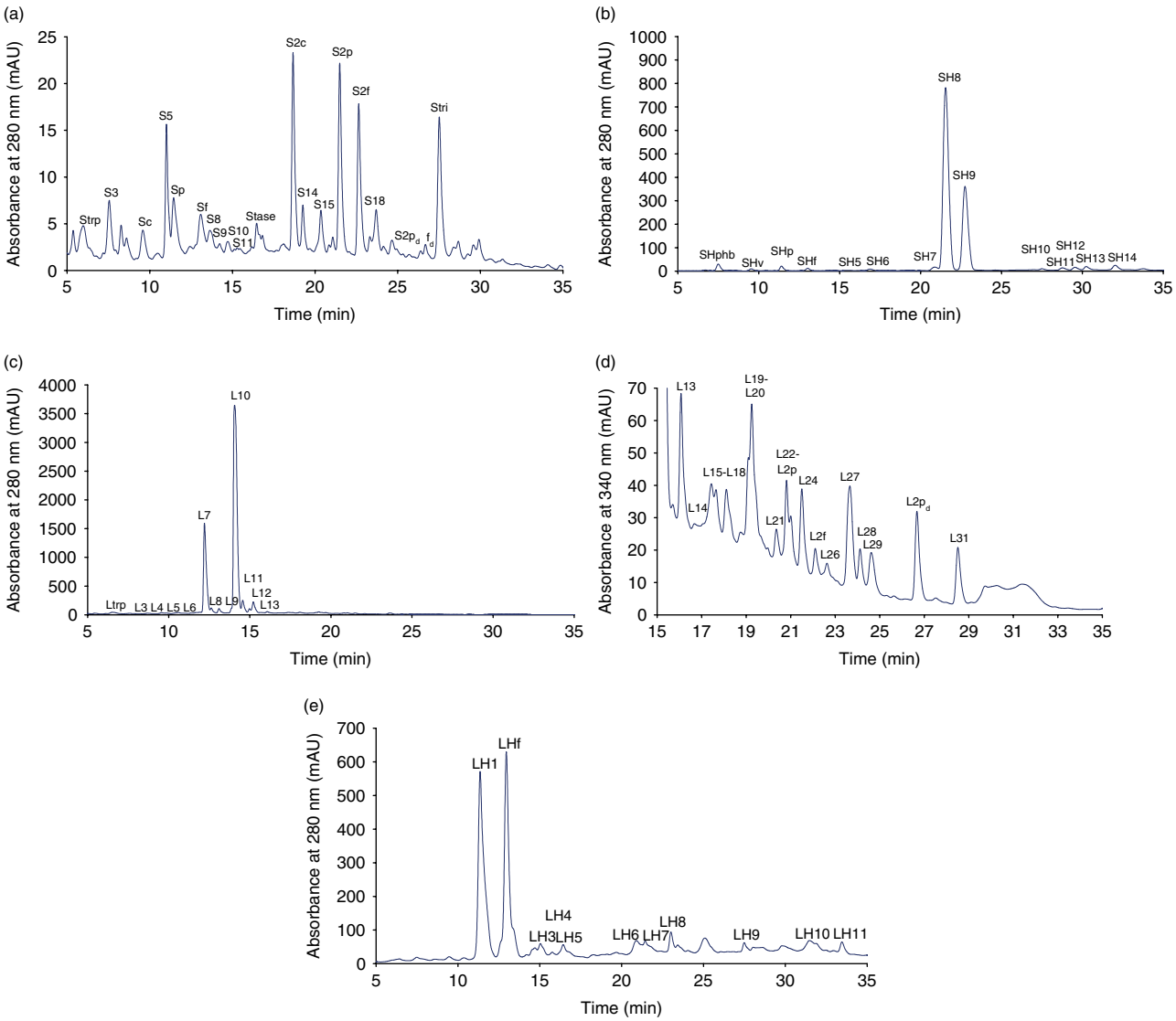

Figure 2. Typical HPLC chromatograms of extracts from non-hydrolysed (a, c) and alkaline hydrolysed (H) (b, e) oat spikelet (S) (a, b) and leaf (L) (c, e) samples detected at 280 nm. (d) Chromatogram of a non-hydrolysed leaf sample, magnified from 15 to 35 min and detected at 340 nm. For peak nomenclature, see Results section.

5-hydroxyanthranilic acid (dissolved in DMSO and stored at −20 °C) and 10 µL of 12 mmol L^{-1} *p*-coumaroyl-CoA (*p*-CoA) (dissolved in acidic water and stored at −80 °C) were added. The reaction mixtures were vortexed and incubated for 30 min at 30 °C before the enzyme was inactivated by adding 20 µL acetic acid. As a control, acetic acid was added prior to the two substrates, 5-hydroxyanthranilic acid and *p*-CoA, to two aliquots. These controls also revealed that no endogenous avenanthramides were present and detectable in the oat flours. The volume was adjusted to 500 µL by adding MeOH and the samples were centrifuged at 13 000 rpm for 10 min before they were filtered through a 0.45 GHP Acrodisc 13 filter. The samples were subjected to HPLC analyses (see below). The amount of 2p (mmol g^{-1} h^{-1}) produced was used as a measure of HHT activity.

HPLC analyses

HPLC was performed on an HP Series 1100 instrument (Hewlett Packard, Waldbronn, Germany) with a reversed-phase column (HP ODS Hypersil, 5 µm, 4.0 mm × 125 mm) and using a combination of 0.01 mol L^{-1} sodium phosphate buffer containing 5% acetonitrile (A) and acetonitrile (B) as the mobile phases. Injection volume was 10 µL and the flow rate 1 mL min^{-1}. Peaks were manually integrated using the software HP ChemStation Version 05.01.

For the analyses of the free and alkaline-released compounds and for PAL activity measurements, a gradient mobile phase system with 0–30% B in A (pH 2.8) for 30 min and a further 5 min with an isocratic system of 30% B in A for was used. The groat compounds were detected at 280 nm and the leaf compounds at 280 or 340 nm. Known peaks were identified using external standards. Cinnamic acid, the enzymatic product formed in the PAL activity analyses, was detected at 210 nm and was identified and quantified using an external standard.

PO activity analyses were performed using an isocratic mobile phase system consisting of 10% B in A (pH 2.8). The analysis time was 8 min. The peak of the substrate *p*-coumaric acid was detected at 280 nm and was identified and quantified using an external standard.

HHT activity analyses were performed using an isocratic mobile phase system with 15% B in A (pH 2.3). The analysis time was 20 min. The avenanthramide 2p was detected at 340 nm and identified and quantified using an external standard.

Statistical analyses

Principal component analysis (PCA) was performed on data using the software Unscrambler Version 8.0 (Camo A/S, Trondheim, Norway). The data used in the PCA were mean values of duplicate analyses from each of the three replicates. All variables were mean centred and scaled to unit variance. Leave-one-out cross-validation was used for all models. Correlation analysis and analysis of variance (ANOVA) using general linear model and Tukey's pairwise comparison analysis were performed using the software Minitab release 11.12 (Minitab Inc., State College, PA, USA). The level of significance was set to $P < 0.05$.

RESULTS

Figure 2 shows typical HPLC chromatograms of extracts from non-hydrolysed (Fig. 2(a) and (c)) and alkaline-hydrolysed (Fig. 2(b) and (e)) oat spikelet (Fig. 2(a) and (b)) and leaf samples (Fig. 2(c) and (e)), respectively. Figure 2(d) represents the 15–35 min portion of a chromatogram of a non-hydrolysed leaf sample, detected at 340 nm with a greatly magnified scale. From the non-hydrolysed spikelet (S) and leaf (L) samples 21 and 31 chromatographic peaks were chosen, respectively. From the hydrolysed samples 14 peaks were chosen for the spikelets (SH) and 11 peaks for the leaves (LH). As the identities of most of the compounds are unknown, the peaks are named S1–S21 and SH1–SH14 for the spikelet samples and L1–L31 and LH1–LH11 for the leaf samples. Compounds that previously have been identified from oat grains are tryptophan (trp), *p*-hydroxybenzaldehyde (phb), vanillin (v), caffeic acid (c), *p*-coumaric acid (p), ferulic acid (f), the lignan tase and the avenanthramides 2p, 2c and 2f.[7,8,25] The compounds corresponding to peaks S19, S20 and S21 were isolated in the present study and were, by NMR and LC-MS analyses and in comparison with published data,[5,43] identified as the avenanthramides *N*-(4′-hydroxy-(E)-avenalumoyl)-5-hydroxyanthranilic acid ($2p_d$) and *N*-(4′-hydroxy-3′-methoxy-(E)-avenalumoyl)-5-hydroxyanthranilic acid ($2f_d$) (Fig. 1) and the flavone tricin (tri), respectively. In comparison with the retention time, UV spectrum and molecular weight (LC-MS) of an authentic compound, peak L1 corresponded to the amino acid phenylalanine (Lphe) (Lphe appeared before 5 min and is not shown in the chromatogram). All the identified compounds are named using an abbreviation instead of a number. Some peaks were not completely separated and were thereby difficult to integrate. These peaks are treated and reported as one peak.

Almost all analysed peaks in the respective chromatograms (spikelets or leaves, non-hydrolysed or hydrolysed) were present in all samples to some degree. An exception was peak S5 in the non-hydrolysed panicle samples (Fig. 2(a)). The compound corresponding to this peak was present in about the same amount in Bay (s), Gem (ir), Vista (ir), Belle (r) and X7571-1 (r), but was not detected in Ogle (s), Dane (is), Jim (is) and Moraine (ir) (not shown).

Comparing retention times and UV spectra, it was found that four compounds, trp and the avenanthramides 2p, 2f and $2p_d$, were detected in non-hydrolysed samples of both spikelets and leaves. Among these compounds, only trp showed a significant correlation between the amounts present in the spikelet samples and their corresponding leaf samples ($R = 0.868$; $P < 0.000$). The mean value of Ltrp in all leaf samples was approximately 13-fold higher than the mean value of Strp in the spikelet samples (1477 nmol g^{-1} *versus* 110 nmol g^{-1}, $P = 0.001$). The cinnamic acid derivatives p and f were found in both non-hydrolysed and hydrolysed spikelet samples, and f was also found in hydrolysed leaf samples. In the spikelets the concentrations of p and f were on average about 8 times higher in the extracts of the hydrolysed as compared to the non-hydrolysed samples (not shown). However, the differences in p and f content between the hydrolysed and non-hydrolysed samples might be even greater, as peaks SH8 and SH9 (Fig. 2(b)) might be methylated forms of p and f. These peaks had UV spectra very close to p and f, respectively, and LC-MS analyses revealed that the molecular weights [M + 1] were 179 and 209, respectively, which correspond to the molecular weights of methylated p and f. Initially, to hydrolyse bound phenols from ground spikelets, the samples were incubated in 3 mol L^{-1} KOH (dissolved in water) at 50 °C for 60 min. This resulted in the formation of a strong gel. To avoid this gel formation a solution of 1 mol L^{-1} KOH in MeOH was used for hydrolysis instead, and this might have produced the methylated forms of p and f. The concentrations of cinnamic acid derivatives were not correlated among the non-hydrolysed spikelet, hydrolysed spikelet and hydrolysed leaf samples.

Enzymatic activities of PO and PAL were detected in both groat and leaf samples, but HHT activity was detected only in the groat samples. A significant negative correlation between PAL activity in the groats and activity in the corresponding leaves was found ($R = -0.652$; $P = 0.041$). On average, the PAL activity in the leaves was approximately two times higher than in the groats (59.1 nmol g^{-1} h^{-1} *versus* 25.4 nmol g^{-1} h^{-1}, $P = 0.001$). No such correlation was found for the PO activity.

To determine the contribution of the various chromatographic peaks in the spikelet or leaf samples to the variations found among the genotypes, PCA was performed (Figs 3 and 4). The total variation explained by the first two principal components of the spikelet samples was 54% (Fig. 3). The loading plot (Fig. 3(A)) shows that most of the compounds released by hydrolysis were associated with each other (upper right quadrant), and that they were separated from the free compounds (except from Strp and Stri). The free compounds fell into three groups: a small group consisting of S3, Sc, Sp and S11, mostly in the upper left quadrant, a larger group mostly in the lower right quadrant, and the two compounds that fell within the SH group. The score plot (Fig. 3(B)) shows that there were differences between the genotypes. When comparing the loading plot with the score plot it was possible to conclude that the more rust-resistant genotypes (Gem, Vista, Moraine, Belle, X7571-1) in principal had higher content of most of the free compounds than the more susceptible genotypes (Bay, Ogle, Dane, Jim). The PCA plots also show that there was a large variation between replicates. The genotypes that differed the most due to replicates were Belle (r) and X7571 (r). No specific compound appeared to be responsible for the replicate derived genotype differences in the spikelets. Rather, a group of the free compounds may have small, but additive effects, with higher total content in replicate 1.

To statistically guarantee the relationships between the various chromatographic peaks and the rust score, correlation analyses were performed. The analyses of the spikelet samples revealed that Strp, S3, Sc and Sp showed a significant positive correlation and S8, Stase, S2c, S14, S2p and $S2p_d$ a significant negative correlation to the crown rust score (Table 2). When grouping the genotypes corresponding to their inherent resistance to crown rust infection (Table 1), i.e. the most crown rust-resistant genotypes (Gem, Vista,

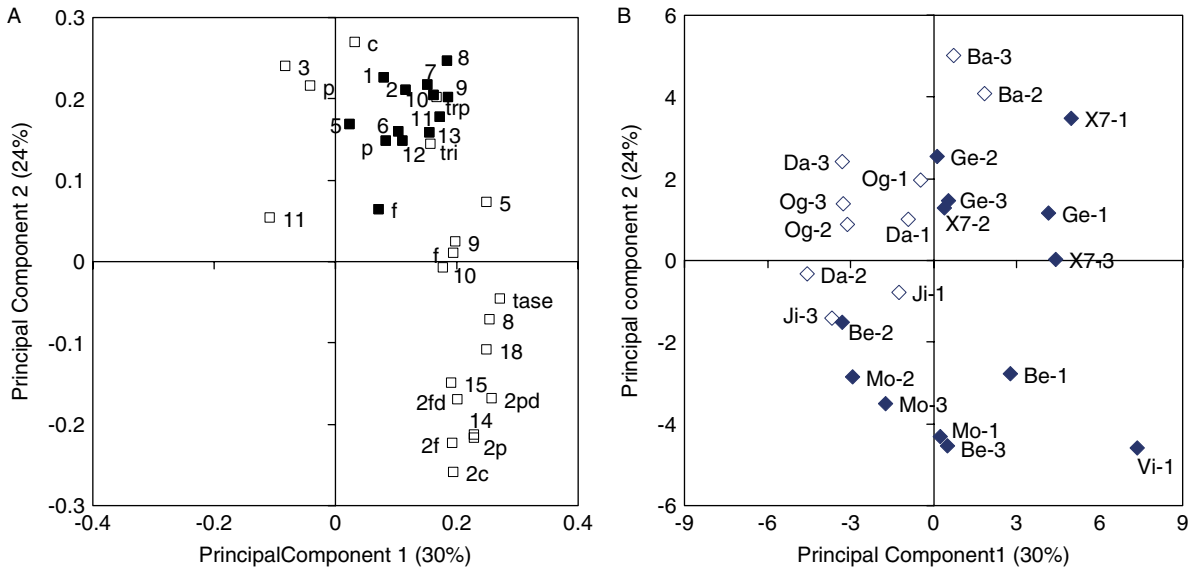

Figure 3. Loading (A) and score (B) plots from the principal component analysis (PCA) performed on chromatographic peak areas from spikelet extracts of nine different oat genotype samples: (Bay (Ba), Ogle (Og), Dane (Da), Jim (Ji), Gem (Ge), Moraine (Mo), Vista (Vi), Belle (Be) and X7571-1 (X7) grown in three replicates (1, 2 and 3). The symbols □ and ■ in A correspond to the free (S) and alkaline released (SH) spikelet compounds, respectively. The symbols ◊ and ♦ in B correspond to the most crown rust-susceptible (Ba, Og, Da, Ji) and the most crown rust-resistant (Ge, Vi, Mo, Be and X7) genotypes, respectively. Missing samples: Ba-1, Ji-2, Vi-2 and Vi-3.

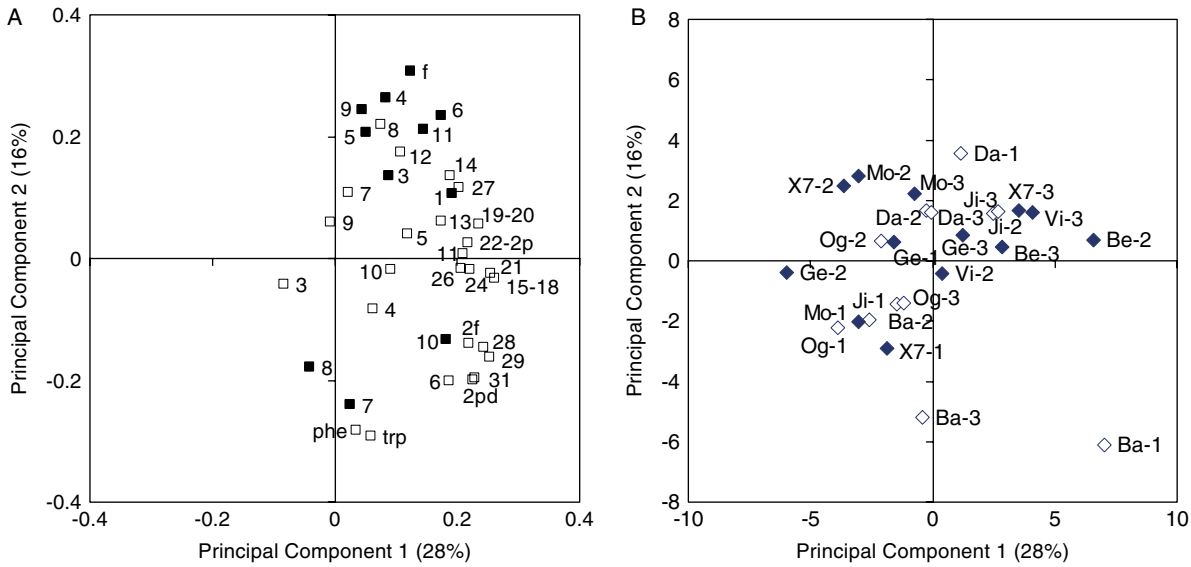

Figure 4. Loading (A) and score (B) plots from the principal component analysis (PCA) performed on chromatographic peak areas from leaf extracts of nine different oat genotype samples: Bay (Ba), Ogle (Og), Dane (Da), Jim (Ji), Gem (Ge), Moraine (Mo), Vista (Vi), Belle (Be) and X7571-1 (X7) grown in three replicates (1, 2 and 3). The symbols □ and ■ in A correspond to the free (L) and alkaline released (LH) leaf compounds, respectively. The symbols ◊ and ♦ in B correspond to the most crown rust-susceptible (Ba, Og, Da, Ji) and the most crown rust-resistant (Ge, Vi, Mo, Be and X7) genotypes, respectively. Missing samples: Vi-1 and Be-1.

Moraine, Belle, X7571-1) in one group and the most crown rust-susceptible ones (Bay, Ogle, Dane, Jim) in another group, Turkey's pairwise comparison analysis revealed that the spikelet compounds S5, Stase, S2c, S14, S2p, S2f, S18 and S2p$_d$ were all significantly higher in the resistant genotype group compared to the susceptible group (Table 2). All these compounds also correlated positively to each other. The unknown compound S3 was present in the highest amounts in the susceptible group. None of the compounds released by alkaline hydrolysis of the spikelet samples were significantly correlated to the rust infection in these analyses (not shown).

No differences in PAL activities of groats were found among the different genotypes. There was no correlation between PAL activity and crown rust score and no significant differences in PAL activity between the crown rust-resistant group (Gem, Moraine, Vista, Belle and X7571-1) and the susceptible one (Bay, Ogle, Dane and Jim) (Table 3). The PO activities in the groats were significantly lower in the genotypes Jim (is) and Vista (ir) compared to the activities in the other genotypes (not shown). There was no significant correlation found between PO activity and crown rust score, and no significant differences between the two genotype groups (see

Table 2. Compounds in the spikelet and leaf samples found to show significant correlations (positive or negative) to the crown rust score (Table 1) and/or found to show significant differences in concentration when comparing the most crown rust-resistant genotype group (Gem, Vista, Moraine, Belle and X7571-1) with the most crown rust-susceptible genotype group (Bay, Ogle, Dane, and Jim)

Compound	Correlation between peak area g^{-1} and rust score (R values)	Compound	Most crown rust-resistant genotype group (mean peak area $g^{-1} \pm$ SE)[b]	Most crown rust-susceptible genotype group (mean peak area $g^{-1} \pm$ SE)
Spikelets[a]		*Spikelets*		
Strp*[c]	$R = 0.491, P = 0.017$	Strp	296 ± 36	360 ± 42
Sc*	$R = 0.499, P = 0.015$	Sc	191 ± 12	222 ± 22
Sp**	$R = 0.590, P = 0.003$	Sp	371 ± 20	501 ± 50
Stase**	$R = -0.552, P = 0.006$	Stase**	185 ± 19	82 ± 12
S2c**	$R = -0.625, P = 0.001$	S2c**	439 ± 49	164 ± 17
S2p*	$R = -0.505, P = 0.014$	S2p**	496 ± 63	167 ± 26
S2f	$R = -0.330, P = 0.124$	S2f*	325 ± 53	142 ± 26
$S2p_d$*	$R = -0.482, P = 0.023$	$S2p_d$**	101 ± 14	37 ± 5
S3***	$R = 0.720, P < 0.000$	S3*	277 ± 22	408 ± 54
S5	$R = -0.376, P = 0.077$	S5**	321 ± 56	68 ± 46
S8*	$R = -0.423, P = 0.044$	S8	107 ± 19	67 ± 8
S14*	$R = -0.455, P = 0.029$	S14**	147 ± 19	64 ± 5
S18	$R = -0.341, P = 0.111$	S18*	451 ± 42	286 ± 25
Leaves[d]		*Leaves*		
Lphe**	$R = 0.644, P = 0.001$	Lphe	4681 ± 1014	9240 ± 2315
Ltrp***	$R = 0.765, P < 0.000$	Ltrp	13237 ± 2352	30795 ± 6942
L8**	$R = -0.629, P = 0.001$	L8	4022 ± 293	2855 ± 331
L27*	$R = -0.486, P = 0.014$	L27	201 ± 41	118 ± 14

[a] Missing samples: Ba-1, Ji-2, Vi-2, Vi-3.
[b] Mean of three replicates.
[c] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
[d] Missing samples: Vi-1, Be-1.

Table 3. Correlations between enzyme activities and rust score (Table 1) and differences in activities when comparing the most crown rust-resistant genotype group (Gem, Vista, Moraine, Belle and X7571-1) with the most crown rust-susceptible genotype group (Bay, Ogle, Dane, and Jim)

Enzyme activity	Correlation between enzyme activity and rust score (R and P-values)	Enzyme activity	Most crown rust-resistant genotype group (activity $\pm$ SE)[a]	Most crown rust-susceptible genotype group (activity $\pm$ SE)
Groats		*Groats*		
PAL[b] (nmol cinnamic acid produced g^{-1} h^{-1})	$R = -0.177$ $P = 0.482$	PAL	27.3 ± 2.1	20.7 ± 1.9
PO[c] (nmol *p*-coumaric acid produced g^{-1} h^{-1})	$R = -0.069$ $P = 0.760$	PO	1153 ± 151	1048 ± 134
HHT[b] (nmol **2p** produced g^{-1} h^{-1})	$R = -0.305$ $P = 0.192$	HHT*[f]	241 ± 11	179 ± 25
Leaves		*Leaves*		
PAL[d] (nmol cinnamic acid produced g^{-1} h^{-1})	$R = 0.427$ $P = 0.128$	PAL	52.3 ± 7.3	68.2 ± 7.8
PO[e] (nmol *p*-coumaric acid produced g^{-1} h^{-1})	$R = -0.096$ $P = 0.671$	PO	538 ± 53	617 ± 126

[a] Mean of three replicates.
[b] Missing samples: Ba-1, Ba-3, Og-3, Da-3, Ji-2, Vi-2, Vi-3.
[c] Missing samples: Ba-1, Ba-3, Ji-2, Vi-2, Vi-3.
[d] Missing samples: Replicate 1, Og-2, Og-3, Vi-2, Be-3.
[e] Missing samples: Og-3, Ji-1, Mo-1.
[f] * $P < 0.05$.

above) (Table 3). The groat HHT activities did not differ among genotypes. No significant correlation between HHT activity and the rust score was found, but the HHT activity was significantly higher in the rust-resistant genotype group (see above) compared to the susceptible group. HHT activity was positively correlated with Stase, S2c, S2p and S2f (R values 0.49–0.57; $P < 0.05$).

The two first principal components from the PCA performed on the chromatographic peaks of the leaf samples explained 44% of the total variance (Fig. 4). The free compounds were not grouped and separated from those released by hydrolysis in the leaves as was found in the spikelet loading plot. Genotype differences were found in both PC 1 and PC 2, but it was not possible to assign any specific compounds which explained genotypic differences

due to their inherent resistance to crown rust (Fig. 4). Among the replicates Bay (s), Jim (is), Gem (ir) and X7571-1 (r) differed the most, and the compounds mainly responsible for this difference were L3, L10, L21, LH7 and LH8, which in general were present in significantly higher concentrations in replicate 1.

A correlation analysis of the data of the leaf samples showed that the two amino acids, Lphe and Ltrp, correlated positively and that two compounds, L8 and L27, correlated negatively with crown rust infection (Table 2). Furthermore, a positive correlation was found between the two amino acids Lphe and Ltrp ($R = 0.918$; $P < 0.000$) and between L8 and L27 ($R = 0.432$; $P = 0.031$) and a negative correlation was found between Lphe and L8 ($R = -0.504$; $P = 0.010$) and between Ltrp and L8 ($R = -0.626$; $p = 0.001$). When grouping the genotypes corresponding to their inherent rust resistance (see above), Tukey's pairwise comparison analysis showed that none of the leaf compounds, free or hydrolysed, were significantly more abundant in either of the groups.

There was no difference in PAL or PO activities in the leaf samples among the genotypes. The activities were not correlated to crown rust score, and no differences were found when the mean activity values of the crown rust-resistant genotype group were compared with the mean values of the susceptible group (Table 3). PAL activity did not correlate with the content of its substrate phenylalanine (Lphe). HHT activity was not detected in any of the leaf samples.

DISCUSSION

Studies have suggested that enhanced PAL, PO and peroxidase activities and biosynthesis of phenolic compounds are involved in resistance and in induced defence mechanisms of various plant species against penetration by attacking pathogens.[30–32] The present study showed that the spikelets of the crown rust-resistant oat genotypes in general had higher content of the free compounds than the more susceptible genotypes and that Stase and several avenanthramides were the most distinguishing compounds. The avenanthramides S2c, S2p and $S2p_d$ were negatively correlated with the rust infection score, and other avenanthramides, S2f and $S2f_d$, were also more abundant in the crown rust-resistant group compared to the susceptible group. The unknown compounds S14 and S18, which also were positively related to the resistant genotypes may, due to their chromatographic retention times and UV spectra, very well be avenanthramide compounds. This has to be confirmed, however. The resistant genotype group also had higher activity of the enzyme HHT. This result seems logical as HHT is involved in the biosynthesis of avenanthramides.[10,12,35,36] In contrast to the spikelet findings and to the previous findings in leaves of glasshouse-grown oat plants,[9,10,12,35,36] no significant negative correlations were found between avenanthramides in the leaves and crown rust infection. Instead, other compounds, especially L8 and L27, correlated negatively with the crown rust score. The identities of these compounds are not known. In leaves as well as spikelets, no differences in PAL and PO activities were found between the crown rust resistant and susceptible groups.

In the present study it was not possible to tell whether the higher contents of Stase and the avenanthramides in the spikelet samples and L8 and L27 in the leaf samples of the resistant group were induced by an attack by crown rust fungi. This phenomenon has been described in leaves of young greenhouse-grown oats.[9,10,12,35,36] However, since PAL and PO activities were not associated with crown rust infection in the plant and since these enzymatic activities were the same in the resistant and susceptible groups, the result indicates that no induction occurred. One explanation might be that PAL and PO activities have been induced by the fungi in the resistant genotypes at an earlier stage of development, resulting in higher content of various phenols, but that the activities at maturity have returned to basic level. Alternatively, higher levels of certain phenols may already exist in the spikelets and leaves of the genotypes that carry genes for rust resistance before they were exposed to the fungus. Thus high amounts of endogenous Stase and avenanthramides in the grains and of L8 and L27 in the leaves may be markers of highly resistant oat genotypes. There could also be a difference between field-grown plants and plants grown in a glasshouse. It might be suggested that in the field the plants have been influenced by biotic and abiotic stresses in addition to crown rust, and these factors may have increased the level of enzyme activities and the content of various phenolic compounds in the crown rust-susceptible varieties as well. This is in agreement with the study performed by Peltonen,[45] who showed that PAL activity did not increase in barley leaves that had been exposed to a non-pathogen prior to a subsequent challenge with a pathogen, whereas the activity in non-induced plants was increased. Furthermore, Shiraishi *et al*.[46] showed that PAL activity increased in barley leaves regardless of resistance or if the plants were inoculated with a pathogen or a non-pathogen, suggesting that the PAL activity response was non-specific. In a very recent study Wise *et al*.[47] measured the avenanthramide concentration in grains of 18 oat genotypes with different inherent resistance to crown rust, grown at six different field environments. They found that avenanthramides accumulated more in resistant genotypes that were grown in environments with high fungal pressure than in the same genotypes grown in fungal-free environments or in susceptible genotypes regardless of environment. These results indicate that avenanthramide production was induced in the resistant genotypes.

The peak S5, which from its UV spectrum appears to be a flavanoid,[48] was also over-represented in oat genotypes that were more resistant to crown rust. However, S5 was also present in the highly susceptible genotype Bay. It is interesting to note that Bay was resistant to crown rust when it was released in 1995[49] but now has become highly susceptible. Nevertheless, if not a marker for resistance, S5 seems be a characteristic strongly influenced by genotype.

The content of the compounds Lphe and Ltrp in the leaves and Strp, S3, Sc and Sp in the spikelets were all positively correlated with rust infection. This was mainly attributed to the high content of these compounds in the genotype Bay, which was heavily infected. The amino acids phenylalanine and tryptophan in the leaves may come from the fungus itself or may result from protein digestion during fungal penetration of the plant. However, it may be harder to propose the same explanation for the spikelets, because crown rust did not infect the groats. (M Carson, personal communication). The fungi were present on the empty glumes, however, and those tissues were included in the spikelet analyses, so it might be reasonable to infer that the main part of the actual amino acids were also of fungal origin or a result of fungal activities in the spikelets. The same may be valid for S3, Sc and Sp. Wang and co-workers[50,51] found that the amino acid concentration in wheat flours was positively correlated with the severity of fungal infection and that fungicide treatment decreased the amino acid concentration in the flours. Wheat kernel threshes free of empty glumes, lemma and palea; i.e., it is similar to the oat groat.

However, rust infection of vegetative plant parts may produce free amino acids and other compounds that are transported into the developing grains. The concentration of the flavone tricin (Stri) was also highest in Bay, but this compound was not correlated with rust infection.

It has been shown that the tryptophan is acting as a feedback inhibitor towards anthranilate synthase, the enzyme involved in the synthesis of anthranilic acid, which is a precursor for the synthesis of avenanthramides.[52] One might thereby expect negative correlations between Strp or Ltrp and the avenanthramides, but no such correlations were found.

The identities of most of the compounds released by alkaline hydrolysis are unknown, but from literature data it may be assumed that, besides *p*-hydroxybenzaldehyde (phb), vanillin (v), and *p*-coumaric (p) and ferulic (f) acids several dehydrodimers of p and f and of various avenanthramides were released from the cell walls.[41,53] PO activity was positively correlated with eight of the phenols released by hydrolysis in the grains, and with only two of the free compounds (not shown). This could be an indication that PO activity is important and may be involved in the dimerization processes of the cinnamic acid derivatives, which precedes their incorporation into the cell walls. Oxidatively coupled dehydrodimers of cinnamic acid derivatives play a role in lignification and cross-linking of cell wall polymers and are important structural components in plant cell walls.[41,54] They serve to enhance rigidity, strength, and mechanical and chemical protection of the plant. However, none of the compounds released by hydrolysis in the present study, either in the spikelets or in the leaves, correlated with rust infection and thereby could not be related to fungal protection.

From the PCA score plot it seemed as if the spikelet samples in replicate 1 had higher contents of most of the free compounds than samples from the other replicates. Even though the plants were cultivated in the same field, the variation between the replicates could be due to different microenvironments and the plants may have been exposed to different levels of biotic and abiotic agents during growth in the field. In fact, the mean rust score in replicate 1 was significantly lower than the scores in the other replicates (Table 1). It has been shown in several studies that the phenolic compounds in oat grains are affected by genotype and also by cultivation conditions, location and growing season.[55–59] The three replicates varied more in the leaf samples than in the spikelet samples, indicating that the chemical composition is more prone to changes in response to environmental influences in the leaves compared to the spikelets.

The spikelet samples were a combination of several tissues: the photosynthetic modified leaves (empty glumes, lemma and palea) and the developing caryopsis (embryo and endosperm). With maturation, the caryopsis (groat) becomes the predominant tissue in these samples. Therefore, the spikelet samples in this study had less exposed surface susceptible to infection than did the leaves per unit weight. The groats were protected by the surrounding tissues and not directly infected. Because different compounds are present in the spikelets compared to the leaves and because spikelets and leaves seem to react differently to the environmental conditions, it is not possible to predict the chemical composition or enzymatic activities in the spikelets by studying the leaves. Nevertheless, because crown rust-resistant oat genotypes seemed to have higher endogenous levels of health-promoting phytochemicals such as avenanthramides in the grains, use of resistant genotypes may contribute to a raw material for food with potential health beneficial effects. This would be in addition to the agronomic benefits of planting resistant genotypes, i.e. higher yield, better quality, reduced use of agrochemicals.

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